

Suppression of the Neutral Protease Activity of Macrophages Treated with Asbestos *in Vitro*

by John A. Hamilton*

Macrophages are often conspicuous in asbestos-induced inflammatory lesions. Chrysotile type B elicits macrophages into the peritoneal cavity of mice which produce high levels of the neutral protease, plasminogen activator; *in vitro* addition of these same fibers to mouse peritoneal macrophages stimulates enzyme production. It is reported here that, for endotoxin-elicited mouse peritoneal macrophages fed chrysotile type B *in vitro*, the increased plasminogen activator activity is suppressed by low concentrations of anti-inflammatory steroids. Other active drugs include colchicine and vinblastine. These studies are considered important, as they suggest an approach to controlling the levels of a potentially deleterious enzyme system (PA-plasmin) from macrophages treated with asbestos fibers.

Introduction

Asbestosis is characterized by inflammation and fibrosis surrounding the respiratory bronchioles and alveoli and leads to permanent impairment of lung function (1). Macrophages are found in normal alveoli and are the prominent cell type found in chronic inflammatory lesions, including those induced by asbestos (2). Macrophages are able to release *in vitro* a number of molecules which are probably important in the initiation and maintenance of an inflammatory reaction, for example, neutral proteases, complement components, endogenous pyrogens, oxygen metabolites, prostaglandins and analogs, proliferation factors for neighboring cells, etc. (3).

For monocyte macrophages *in vitro*, the activity of the neutral protease, plasminogen activator (PA), shows positive correlations with stimuli [for example, asbestos (4), endotoxin (5), lymphokines (6), and phorbol esters (7,8)] and suppressive drugs [for example, glucocorticoids (9)] associated with an inflammatory response. A role for macrophage PA activity in cell migration from blood to tissue and in the processes of tissue destruction and activation of several inflammatory enzyme cascades has previously been

proposed (9). We have been able to show that IARC chrysotile type B elicits macrophages into the peritoneal cavity of mice which produce high levels of PA (4, 10); *in vitro* addition of these fibers to mouse peritoneal macrophages stimulates enzyme production.

The present studies indicate that the *in vitro* stimulation of macrophage PA activity can be suppressed by anti-inflammatory glucocorticoids and, under certain culture conditions, by colchicine and vinblastine.

Materials and Methods

Reagents

Reagents were obtained commercially as follows: fetal bovine serum (Flow Laboratories, Hamden, CT); asbestos, IARC chrysotile type B, a gift from Johns-Manville Corp.; soybean trypsin inhibitor and bovine fibrinogen; fraction I (Miles Laboratories Inc., Kankakee, IL) lactalbumin hydrolyzate (Nutritional Biochemicals Corp., Cleveland, OH); polystyrene latex particles, 1.01 μ m diameter (Dow Diagnostics, Indianapolis, IN); steroids and colchicine (Sigma Chemical Co., St. Louis, MO); vinblastine (Eli Lilly and Co., Indianapolis, IN).

All steroids were dissolved at 10^{-3} M in ethanol and stored at 4°C. Controls for the effect of solvent never differed from those of other untreated drug-

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free cultures. Plasminogen-free fibrinogen for the fibrin plates was prepared as before (9). Endotoxin, *S. minnesota* lipopolysaccharide, mR595 (S418), was a gift from Dr. O. Luderitz, Max Planck Institut für Immunobiologie, Freiburg, West Germany. Human lysozyme was a gift from Dr. E. Osserman (College of Physicians and Surgeons of Columbia University, New York).

Stock suspensions of asbestos in PBS and latex particles in Dulbecco's medium (DB) at 5×10^9 particles/mL were irradiated with ultraviolet light and kept at 4°C until use (4).

Peritoneal Cells

Peritoneal cells were obtained from NCS (Rockefeller) mice (25–30 g) or BDF₁ mice, 8–12 weeks old (Cumberland Farms), that had been injected intraperitoneally 4 days previously with phosphate-buffered saline (PBS), pH 7.4, or with 30 µg endotoxin (4).

Fibrinolysis Measurement

The peritoneal cells (2×10^6) were plated on ¹²⁵I-fibrin-coated wells (Linbro Dispo Trays, FB 16-24TC) (9) for 24 h in DB supplemented with 5% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS). At this stage, soybean trypsin inhibitor (STI) (100 µg/mL) was added to block any fibrinolysis. The cells were washed twice with PBS and placed again in DB + 5% FBS + STI. The particulate preparations were resuspended by passage through a syringe and 26G needle and carefully distributed in the culture medium. The particles were generally added for 2 hr, after which time >95% of the cells contained 20–50 latex particles. The macrophages were then washed three times with PBS and placed in DB + 5% acid-treated FBS (ATFBS) for assay. The acid treatment removes interfering protease inhibitors (9). Fibrinolysis was estimated by withdrawing aliquots of medium and counting the radioactivity in a Packard Autogamma scintillation spectrometer. The plasminogen dependence of the fibrinolytic activity can be shown by assaying in the presence of ATFBS from which the plasminogen has been removed (9).

Measurement of Secreted Plasminogen Activator

Peritoneal cells (2×10^6) were plated in DB + 5% FBS on regular linbro trays. At 24 hr after plating, the cells were washed twice with PBS and placed again in DB + 5% FBS but also in the presence of the particles for an additional 24 hr. After this period, the cultures were washed twice with PBS and placed in DB containing 0.05% lactalbumin hydrolyzate (LH) (9). After 24 hr, the serum-free condi-

tioned media (CM) were collected; also, the cells were washed twice with PBS, lysed by the addition of Triton X-100 (0.2% v/v in water) and scraped from the surface of the dish with a plastic policeman for protein determination.

Serum-free CM were kept frozen at -20°C until assay for PA activity. For this purpose, ¹²⁵I-fibrin-coated wells of Linbro Dispo Trays were used, and the assay mixture contained 2 µg human plasminogen (purified from outdated human plasma) and 10 µL CM in 250 µL 0.1 M Tris chloride, pH 8.1 (9).

All experiments included appropriate controls for all reagents and media. No plasminogen-independent fibrinolysis could be detected when plasminogen was not included in the assay mixture. One unit of PA is defined as the amount that stimulates the release of 10% of the initial radioactivity in 4 hr (9).

Lysozyme

Lysozyme in serum-free CM was assayed by measuring the initial rate of lysis of a suspension of *Micrococcus luteus*, as described by Gordon et al. (11). Human lysozyme diluted in DB + 0.05 % LH was used as standard.

Lysosomal Enzymes

N-Acetyl-β-D-glucosaminidase and β-galactosidase were assayed as described by Bowers et al. (12).

Protein

Protein was measured by the method of Lowry et al. (13) with bovine serum albumin as standard.

Results

Enhanced Macrophage PA Activity after Asbestos Exposure *in Vitro*

Phagocytosis of digestible and nondigestible materials enhances the PA activity of mouse peritoneal macrophages (4, 5). Figure 1 demonstrates that asbestos fibers can increase the PA activity of peritoneal macrophages obtained from endotoxin-injected mice and also PBS-injected mice as a control population. Comparable results were obtained whether the asbestos addition was allowed for 2 hr or for 24 hr prior to the PA measurement. For asbestos doses less than 100 µg, there did not appear to be any gross alterations in cell morphology, but with 100 µg fiber there was some cell rounding and detachment after 3–4 days. The control, unstimulated peritoneal macrophages released PA activity in the presence of the asbestos, but there was a longer lag period than for

the endotoxin-induced cells. The fibrinolytic activity was predominantly (> 95%) plasminogen-dependent, and hence PA activity was being monitored.

Inhibition of *in Vitro*-Stimulated Fibrinolytic Activity with Drugs

If the assumption is correct that the generation of excessive PA activity around macrophages interacting with asbestos fibers would be deleterious, then it would be desirable to find drugs which might suppress this local proteolysis. In Table 1, a comparison is made of three steroids for their effect on the asbestos-stimulated plasminogen-dependent fibrinolytic activity. The glucocorticoids, dexamethasone and prednisolone, are effective whereas progesterone is not. The data using latex feeding to cells are included for comparison. This inhibition of the increased fibrinolysis persists for at least 72 hr, the maximum assay time.

Table 2 lists the relative efficacies of different steroids. Also included for comparison are colchicine and vinblastine, drugs implicated in the control of secretory processes. Certain glucocorticoids are again effective. It is interesting that the activity profile for the steroids is similar to that found for their suppression of the PA activity of thioglycollate-induced macrophages (9). Colchicine and vinblastine had little influence on the cells if plated on 125 I-fibrin

(Table 2) but were quite effective if harvest fluid is assayed for the PA activity from asbestos-treated cells (see below). It can be noted that the PA activity of the endotoxin-primed cells themselves was also inhibited. From Table 1 and 2, it can be observed that

Table 1. Effect of steroids on the fibrinolytic activity for cells treated with asbestos *in vitro*.

Macrophages (8×10^5) ^a	Drug (10^{-7} M)	Radioactivity released at 24 hr, %
Endotoxin	—	8
	Dexamethasone	0
	Prednisolone	0
	Progesterone	6
Endotoxin + asbestos (100 μ g)	—	34
	Dexamethasone	1
	Prednisolone	2
	Progesterone	26
Endotoxin + latex	—	42
	Dexamethasone	6
	Prednisolone	7
	Progesterone	39

^a Peritoneal cells were cultured for 24 hr on 125 I fibrin-coated Linbro wells in the usual manner, before the asbestos or latex feeding. After washing, the solid particles were added in DB + 5% FBS and 100 μ g/mL STI for 2 hr at 37°C. After washing to remove excess particles, DB + 5% ATFBS was added in presence or absence of drug. Samples assayed as in Fig. 1.

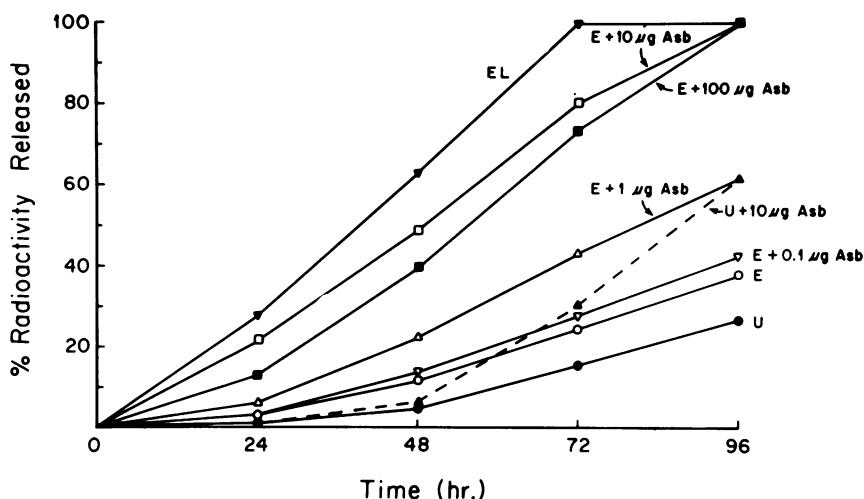


FIGURE 1. Effect of asbestos feeding *in vitro* on macrophage fibrinolysis. Before the asbestos feeding, 2×10^6 peritoneal cells were cultured for 24 hr on 125 I-fibrin-coated Linbro wells. The cells were then washed once with PBS, then various quantities of the solid particles added in 1 mL DB containing 5% FBS and 100 μ g/mL STI for 2 hr at 37°C, the excess particles removed by washing twice with PBS and then 2 mL of DB containing 5% ATFBS added. Samples were aliquoted from duplicate cultures and counted for released radioactivity. (O) endotoxin-stimulated macrophages (E); (▽) E fed 0.1 μ g asbestos (E + 0.1 μ g Asb); (△) E fed 1 μ g asbestos (E + 1 μ g Asb); (□) E fed 10 μ g asbestos (E + 10 μ g Asb); (■) E fed 100 μ g Asb (E + 100 μ g Asb); (▽) E fed latex (EL); (●) unstimulated macrophages (U) (▲) U fed 10 μ g asbestos (U + 10 μ g Asb).

Table 2. Effect of drugs on the fibrinolytic activity for cells tested with asbestos *in vitro*.

Macrophages (8×10^5) ^a	Drug (10^{-7} M)	Radioactivity released at 24 hr, %
Endotoxin	—	24
	Hydrocortisone	8
	9-Fluorocortisone	14
	Desoxycorticosterone	20
	Cortexelone	24
	Aldosterone	22
	Testosterone	23
	β -Estradiol	25
	Colchicine	33
	Vinblastine	35
Endotoxin + 100 μ g asbestos	—	59
	Hydrocortisone	21
	9-Fluorocortisone	25
	Desoxycorticosterone	32
	Cortexelone	53
	Aldosterone	45
	Testosterone	56
	β -Estradiol	56
	Colchicine	51
	Vinblastine	41
Endotoxin + latex	—	54
	Hydrocortisone	17
	9-Fluorocortisone	24
	Desoxycorticosterone	31
	Cortexelone	51
	Aldosterone	44
	Testosterone	44
	β -Estradiol	48
	Colchicine	74
	Vinblastine	46

^a Same experimental protocol as in Table 1.

there is a comparable effect of the drugs for cells fed latex particles.

The most potent glucocorticoid tested was dexamethasone and, as can be seen from Table 3, it is effective even at doses as low as 10^{-9} M. This low dose was also found to be effective on thioglycollate-induced macrophages (9).

Enzyme Secretion after Asbestos Exposure *in Vitro*

The above experiments were all carried out with cells plated directly on the fibrin substrate. Macrophages obtained from endotoxin-injected mice can also secrete elevated PA levels after exposure to asbestos (or latex) *in vitro* (Table 4); the increase is predominantly extracellular (data not shown), indicating that enzyme synthesis and secretion were tightly coupled (9). There was no effect of asbestos (or latex) on the secretion either of lysozyme or of two lysosomal enzymes, *N*-acetyl- β -D-glucosaminidase and β -

Table 3. Dose response for effect of dexamethasone on the fibrinolytic activity due to asbestos feeding *in vitro*.

Macrophages (8×10^5) ^a	Dexamethasone concn, M	Radioactivity released at 24 hr, %
Endotoxin	—	8
Endotoxin + asbestos (100 μ g)	—	34
Endotoxin + latex	—	47
Endotoxin	10^{-9}	1
Endotoxin + asbestos (100 μ g)	10^{-9}	12
Endotoxin + latex	10^{-9}	13
Endotoxin	10^{-8}	1
Endotoxin + asbestos (100 μ g)	10^{-8}	4
Endotoxin + latex	10^{-8}	4
Endotoxin	10^{-7}	1
Endotoxin + asbestos (100 μ g)	10^{-7}	3
Endotoxin + latex	10^{-7}	4

^a Same experimental protocol as for Table 1.

galactosidase (Table 4). Further evidence in support of the concept of differential secretion control for PA and for lysozyme and lysosomal enzymes is the fact that dexamethasone and colchicine, drugs which have a dramatic effect on the extracellular PA levels, fail to alter the levels of lysozyme and lysosomal enzymes from macrophages when they are treated with asbestos or latex particles.

Discussion

Previous studies (4, 10) have indicated that intraperitoneal injection of IARC chrysotile type B into mice induces an inflammatory exudate and that macrophages cultured from such exudates have elevated levels of PA. It was also shown that peritoneal macrophages when treated with the same asbestos fibers *in vitro* are stimulated to produce more PA activity. The possible significance of macrophage PA activity in inflammation has been outlined (9, 10). It has been shown above that as little as 0.1 μ g of the asbestos fibers can raise the PA activity of endotoxin-primed mouse peritoneal macrophages. It is tempting to speculate that, since the *in vitro* responses resemble those found with latex particles, it is phagocytosis of the asbestos fibers which results in the heightened protease activity. However, until a quantitative analysis of fiber uptake is made, such a conclusion is unwarranted, particularly as other agents, such as phorbol esters (7, 8), concanavalin A (7) lymphokines (6) and colony-stimulating factors (14), which are presumably not acting via a phagocytic pathway, can all stimulate macrophage PA activity.

The enhanced PA activity is suppressed by anti-inflammatory glucocorticoids. It might be profitable

Table 4. Effect of drugs on secretion of plasminogen activator, lysozyme and lysosomal enzymes after adding asbestos and latex *in vitro*.

Macrophages (2×10^6) ^a	Drug added ($10^{-7}M$)	Cell protein/dish, μg	Secreted plasminogen activator units/mg cell protein	Lysozyme secreted, $\mu g/mg$ cell protein	N-Acetyl- β -glucos- aminidase secreted		β -Galactosidase secreted	
					$\mu mole/min/mg$ cell protein ($\times 10^3$)	%	$\mu mole/min/mg$ cell protein ($\times 10^3$)	%
Endotoxin	—	130	620	17.6	6.0	40	3.8	46
Endotoxin + asbestos (100 μg)	—	190	2380	17.4	8.1	47	4.1	44
Endotoxin + latex	—	250	2430	15.8	4.4	36	2.0	26
Endotoxin	Dexamethasone	130	240	18.7	8.0	58	4.6	56
Endotoxin + asbestos (100 μg)	Dexamethasone	210	110	16.3	7.3	59	3.5	51
Endotoxin + latex	Dexamethasone	250	280	11.5	3.6	29	1.8	24
Endotoxin	Colchicine	90	140	14.6	10.0	56	4.1	50
Endotoxin + asbestos (100 μg)	Colchicine	100	210	14.6	11.1	75	4.1	69
Endotoxin + asbestos	Colchicine	150	310	10.2	8.3	60	3.4	48

^a Peritoneal cells were cultured in DB containing 5% FBS for 24 hr. The cells were then fed asbestos or latex for 2 hr at 37°C and then, after washing, placed in DB containing 0.05% LH in the presence or absence of the drug. CM and cell lysates were collected after 48 hr.

if more were known about how glucocorticoids are working in this context; perhaps these drugs are inducing a "macroartin-like" molecule which has been proposed to account for the manner in which they suppress cellular prostaglandin production (8, 15). It is again worth noting that, for macrophages, lysozyme and two lysosomal enzymes activities appear to be under different regulatory control to the PA activity.

Studies of carcinogenesis by various forms of asbestos, with and without hydrocarbon carcinogens, have shown that chrysotile can augment considerably the number of malignant tumors produced by benzo[a]pyrene (16, 17): these studies and others have suggested that asbestos fibers can function as cocarcinogens (promoters). Interestingly, we have shown that the tumor promoter in skin, namely, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), and asbestos fibers, elicit similar acute inflammatory responses in rabbit skin (18) and, as mentioned above, can both increase the PA activity of macrophages. We have suggested that vascular alterations due to an inflammatory reaction to both asbestos fibers and to TPA ought to be considered in any analysis of the development of neoplasms resulting from their action (8, 18).

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